# The Nonstructural Protein NSs Induces a Variable Antibody Response in Domestic Ruminants Naturally Infected with Rift Valley Fever Virus

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Rift Valley fever (RVF) is an emerging zoonosis in Africa which has spread to Egypt, the Arabian Peninsula, Madagascar, and Comoros. RVF virus (RVFV) (*Bunyaviridae* family, *Phlebovirus* genus) causes a wide range of symptoms in humans, from benign fever to fatal hemorrhagic fever. Ruminants are severely affected by the disease, which leads to a high rate of mortality in young animals and to abortions and teratogenesis in pregnant females. Diagnostic tests include virus isolation and genome or antibody detection. During RVFV infection, the nucleoprotein encapsidating the tripartite RNA genome is expressed in large amounts and raises a robust antibody response, while the envelope glycoproteins elicit neutralizing antibodies which play a major role in protection. Much less is known about the antigenicity/immunogenicity of the nonstructural protein NSs, which is a major virulence factor. Here we have developed a competitive enzyme-linked immunosorbent assay (ELISA) enabling detection of low levels of NSs-specific antibodies in naturally infected or vaccinated ruminants. Detection of the NSs antibodies was validated by Western blotting. Altogether, our data showed that the NSs antibodies were detected in only 55% of animals naturally infected by RVFV, indicating that NSs does not induce a consistently high immune response. These results are discussed in light of differentiation between infected and vaccinated animals (DIVA) tests distinguishing naturally infected animals and those vaccinated with NSs-defective vaccines.

Rift Valley fever virus (RVFV) is an emerging phlebovirus of the *Bunyaviridae* family (26). It causes a disease which is endemic in sub-Saharan Africa (6, 10, 11, 39) and was recently introduced to the Arabian Peninsula, Madagascar, Mayotte, and the Comoros (1, 2, 8, 9, 33, 35, 36). In humans, RVFV is most commonly associated with a benign febrile syndrome, but in a small number of cases, patients develop ocular symptoms, meningoencephalitis, or a life-threatening hemorrhagic fever. RVFV infection also affects livestock and domesticated animals, causing high morbidity and mortality rates in neonates and young animals as well as abortions or teratogenesis in pregnant animals. Epidemics/epizootics have important economic consequences, not only because of animal mortality but also because embargoes are imposed during outbreaks. There is still no appropriate therapeutic agent or vaccine for humans, and the live attenuated Smithburn modified vaccine commercially available for veterinary use induces abortions or teratogenic effects in vaccinated ewes (39).

Like all bunyaviruses, RVFV has a tripartite genome of negative or ambisense polarity (32, 34). The L and M segments code for the RNA-dependent RNA polymerase and the precursor to the glycoproteins, respectively. The S segment utilizes the ambisense strategy and codes for the nucleoprotein N in the antigenome orientation and for the nonstructural protein NSs in the genomic orientation (12). The NSs protein is the major virulence factor (40). It is a multifunctional protein forming nuclear filaments and acting through several mechanisms. Importantly, it is a strong inhibitor of beta interferon gene activation (4), which maintains the beta interferon promoter in a repressed state through the interaction of NSs with SAP30 and YY1 (21). NSs is also a general inhibitor of cellular transcription, sequestering components of the basic transcription factor TFIIH (18, 20). Additionally, this protein interferes with cellular and viral translation, as it degrades the interferon-induced double-stranded RNA-dependent protein kinase PKR, a ubiquitous protein which suppresses general translation in response to viral infection (13, 15). Moreover, NSs is tightly associated with pericentromeric gamma satellite sequences and induces segregation defects in infected cell nuclei (23). Because of the toxic effects of NSs, the current strategy utilized to develop live attenuated vaccines is based on virus strains defective for NSs either due to spontaneous deletion, as is the case for clone 13 (25), or due to manipulations by reverse genetics (5; for reviews, see references 7 and 14).

RVF diagnosis is classically based on the presence of antibodies against the glycoproteins or the nucleoprotein N. Antibodies directed against the glycoproteins are assessed by seroneutralization tests and play an important role in protection against the disease (27). However, since manipulation of infectious virus requires biosafety level 3 (BSL3) biocontainment, seroneutralization tests are restricted to a few laboratories. As a consequence, several enzyme-linked immunosorbent assays (ELISAs) have been developed, based on either complete inactivated virus antigens or re-

combinant N protein (16, 30, 31), which is the major antigen during most bunyavirus infections, including RVFV infection. Little is known about the immunogenicity of the other viral proteins, such as the polymerase or the nonstructural proteins. An NSs-based ELISA was described for detection of NSs-specific antibodies in experimentally or naturally infected animals and humans (24). However, only a very small number of sera were analyzed, and the authors did not address the question of the robustness of the immune response against NSs in animal populations. Here we developed a sensitive method based on competitive ELISA (cELISA) for the detection of antibodies against NSs and showed that RVFV-positive animals which developed an efficient and high antibody response against the nucleoprotein had a variable response against the NSs protein: approximately 55% elicited an efficient antibody response, while others showed very-low-level or no NSs-specific antibodies. These results are discussed in light of differentiation between infected and vaccinated animals (DIVA) tests distinguishing naturally infected animals and those vaccinated with NSs-defective vaccines.

## MATERIALS AND METHODS

**Cells.** BSR cells, a clone derived from BHK-21 cells, were grown in minimal essential medium (MEM) supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, and 10 mM HEPES. Antibiotics (penicillin and streptomycin) were added.

**NSs antigen.** RVFV NSs protein was expressed via a Semliki Forest virus (SFV) replicon after cloning the NSs open reading frame at the BamHI site of the plasmid pSFV1, as already described (41). The one-step NSs-expressing SFV was activated by trypsin and used to infect BHK-21 cells at a multiplicity of infection (MOI) of 5, as described originally (22). The negative-control antigen consisted of cells infected with SFV expressing no foreign protein. After 18 h of incubation at 37°C, cells were collected and sonicated in 50 mM borate buffer, pH 9.0, containing 120 mM NaCl and 1% Triton X-100.

**Animal antisera against NSs.** The production of mouse antibodies against NSs has already been described (41). Briefly, a recombinant baculovirus expressing NSs of the MP12 strain was generated and used to infect Sf9 cells. The infected cells were incubated at 28°C and collected at 48 h postinfection. Nuclear extract mixed with complete Freund's adjuvant was inoculated intraperitoneally into outbred mice (OF1 mice). Subsequent inoculations were performed on days 21, 42, and 63, using nuclear extract with incomplete Freund's adjuvant.

Rabbits were immunized against a 20-amino-acid peptide corresponding to the exact C terminus of NSs. After 4 or 5 boosters, they were bled and the sera were used for various experiments as already described (21).

**Animal sera.** Sera from sheep, goats, and horned cattle were collected in Djibouti, Mayotte, or Comoros. Another set of 10 IgM-positive sera was obtained during the 2008-2009 outbreak in Madagascar. They were assayed for RVFV-specific antibodies by use of a BDSL kit or a homemade RVFV antigen. Only the positive sera were used in further experiments.

Lambs (6 months old) were vaccinated with the Smithburn attenuated vaccine (provided by Ondersterpoort Biological Products) by the subcutaneous route, using the dose recommended by the producer. The animals were bled at various times postvaccination.

**Procedure for indirect ELISA.** Ninety-six-well plates were coated with the extracts of NSs-expressing cells diluted in phosphate-buffered saline (PBS). Usually, the optimal dilution was 1:400 or 1:500. After incubation with the various dilutions of the tested serum, peroxidase-labeled goat anti-rabbit IgG (AO545; Sigma) or rabbit anti-mouse fragment Fc (Jackson) diluted 1:2,000 was added. The antibody complexes were revealed by the enzyme substrate tetramethylbenzidine in the presence of hydrogen peroxide (Sigma), and the reaction was blocked by the addition

of $H_2SO_4$. The intensity of the enzymatic reaction was measured by determining the optical density at 450 nm ($OD_{450}$).

**Procedure for competitive ELISA.** As for the indirect ELISA, NSs antigen or negative-control antigen was used to coat the wells of reaction plates. First, various dilutions of the tested serum in PBS containing 0.05% Tween 20 were added to the wells and incubated for 75 min at 37°C. After extensive washing, rabbit serum against NSs at a fixed dilution of 1:400 was added to the wells for 45 min at 37°C, the immune complexes were revealed using goat anti-rabbit IgG labeled with horseradish peroxidase (HRP), and the substrate was revealed as in the indirect ELISA. Results were expressed as percent inhibition (PI), for which the following formula was used: PI = [1 − (OD of sample/mean OD of negative serum)] × 100. Each serum was tested in duplicate, and each OD value represents the mean value for the duplicates.

**Western blotting.** Total cell extracts from Vero cells left uninfected or infected with RVFV strain ZH548 or recombinant RVFV deficient in NSs (ZH-DelNSs) (3) were separated in 12% polyacrylamide gels under reducing conditions, transferred to a nitrocellulose membrane, and tested for immunoreactivity with serum samples from field animals, diluted 1:50, or with murine antibodies as a control.

## RESULTS

**Expression of recombinant NSs for ELISAs.** The NSs antigen was produced by infecting BSR cells with a recombinant SFV expressing NSs as previously described (41). This recombinant NSs protein was reported to have the properties of the authentic protein, as it was phosphorylated (19) and formed ribbon-like filaments in the nucleus (37, 38). Lysates from NSs-expressing cells were used as a source of NSs antigen, and the negative control consisted of cells infected with SFV expressing no foreign protein. In a preliminary step, the indirect ELISA was validated using the NSs antigen and an NSs-specific serum prepared by immunizing rabbits with a highly conserved 20-amino-acid peptide corresponding to the exact C terminus of NSs. This hyperimmune rabbit serum has already been used in several experiments involving immunofluorescence, Western blotting, and immunoprecipitation (20, 21). In this assay, the hyperimmune rabbit serum recognized the NSs antigen coated on the wells in a dose-dependent manner, as shown by the titration curve, while only a background level was observed with the negative-control antigen (Fig. 1A). Using this NSs-specific rabbit serum at a dilution of 1:500, we titrated the NSs antigen (Fig. 1B). The curve showed that the NSs antigen gave a significant response at dilutions between 1:200 and 1:500. Therefore, coating of the antigen was performed at a 1:400 dilution in further experiments.

**Competition ELISA.** Although indirect ELISA is a classical technique for the detection of antigen-specific antibodies, the formation of the complex is revealed with a species-specific enzyme conjugate which must be adapted to each animal species studied. cELISA is known to have several advantages over indirect ELISA: (i) since a standardized source of NSs-specific antibodies is utilized to measure the NSs epitopes remaining after competition with the test serum, the assay is animal species independent and always uses the same set of diagnostic reagents; (ii) a high serum concentration can be assayed without increasing the background; and (iii) it is more suitable for poor-quality samples, which may occur with animal sera.

To compare the performances of the competitive and indirect NSs-based ELISAs, we used two NSs-specific sera (sera 9.1 and 11) from mice hyperimmunized with NSs expressed by baculovirus. In the indirect ELISA, the antigen-antibody complexes were revealed with anti-murine IgG antibodies conjugated to peroxidase
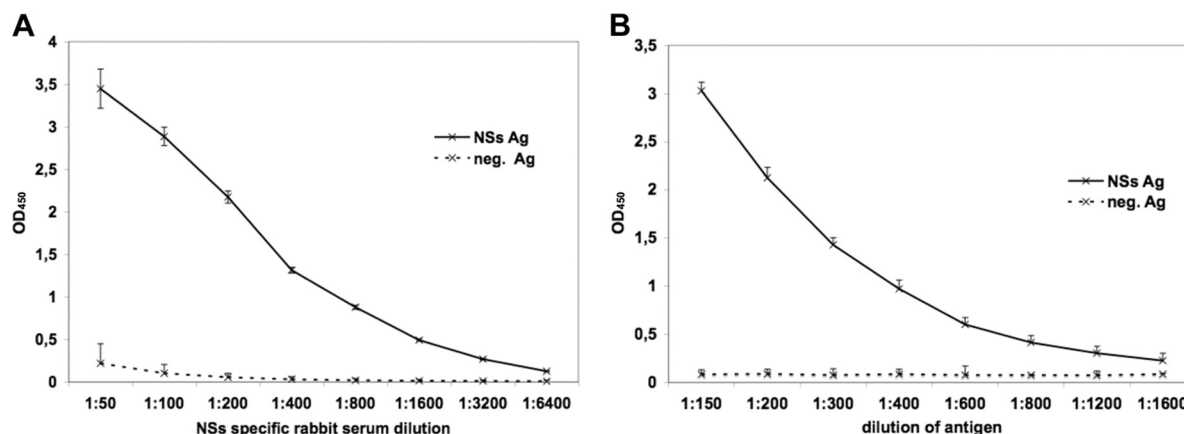
FIG 1 Indirect ELISA for detection of NSs-specific antibodies. (A) Titration of anti-NSs rabbit serum by indirect ELISA. Lysates of cells infected with SFV expressing RVFV NSs (NSs Ag) or no foreign protein (neg. Ag) were used to coat a 96-well plate. After blocking, the wells were incubated with serially diluted NSs-specific rabbit serum. After washing and incubation with goat anti-rabbit antibodies conjugated with HRP, the substrate was added and the optical density was measured. (B) Titration of NSs antigen by indirect ELISA. Serial 2-fold dilutions of NSs antigen (or negative antigen) were used to coat a 96-well plate. Reactions were carried out with the NSs-specific rabbit serum used at a dilution of 1:500. The immune complexes were revealed with a goat anti-rabbit IgG conjugated with HRP.

(Fig. 2A and B). In the cELISA (Fig. 2C), the previously described anti-NSs-peptide rabbit serum and a peroxidase-labeled goat anti-rabbit IgG were used to reveal the epitopes remaining free after reaction with the test serum. In the indirect ELISA, the mouse serum reacted in a dose-dependent manner, but at high serum concentrations, the negative antigen generated a significant background dependent on the quality of the serum. This can mask specific antibodies present at low levels. In the cELISA, the mouse serum was able to compete for binding to the NSs epitopes over a large range of dilutions, and as expected, the percentage of inhibition diminished as the serum dilution increased (Fig. 2C). In addition, only a low background level was observed with the negative antigen (not shown), indicating the specificity of the response for the NSs antigen. These data indicate that both methods are sensitive for the detection of NSs-specific antibodies but that cELISA enables testing of higher concentrations of serum.

**Detection of NSs-specific antibodies in sera of RVFV-infected animals.** To determine if naturally infected animals de-
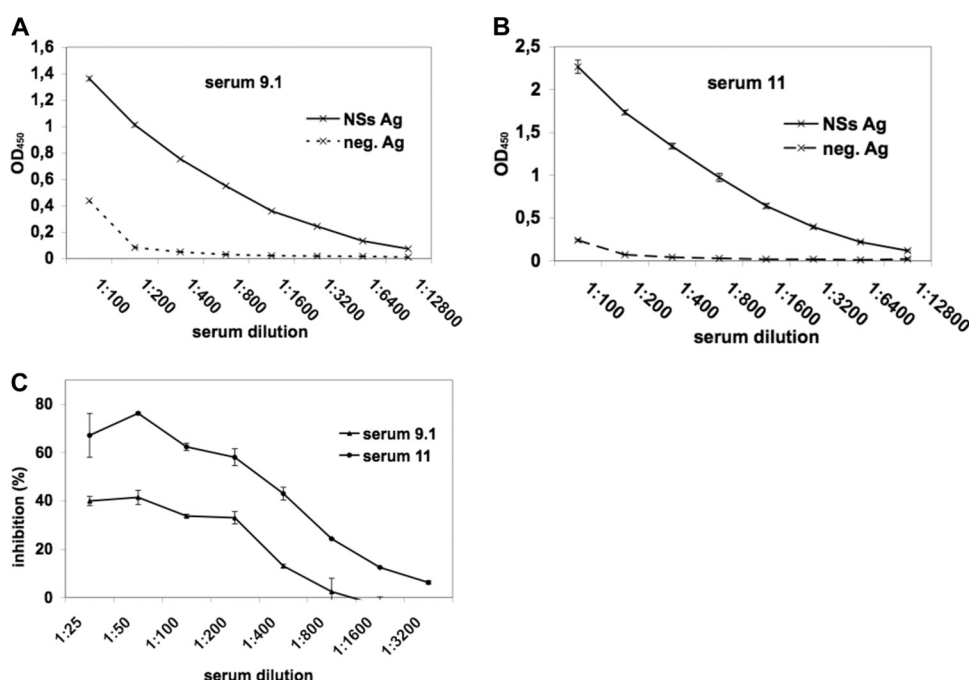


FIG 2 Comparison of indirect and competitive ELISAs. The NSs antigen (or the negative antigen) was used to coat 96-well plates, and two NSs-specific mouse sera, serum 9.1 (A) and serum 11 (B), were titrated using 2-fold serial dilutions. The immune complexes were revealed with rabbit anti-mouse antibodies conjugated to HRP. (C) The NSs antigen was first incubated with serial dilutions of NSs-specific mouse serum (serum 9.1 or 11), and after extensive washing, the remaining free NSs epitopes were titrated with NSs-specific rabbit antibodies, which were revealed with the anti-rabbit HRP conjugate.

**TABLE 1** Antibody responses against NSs among RVFV-positive domestic animals, assessed by cELISA

| Locality or group | Species | No. of positive animals/total no. of animals (%)[a] |
|---|---|---|
| Djibouti | Bovine | 5/11 (45.5) |
| Mayotte | Bovine | 5/7 (71.4) |
|  | Goat | 0/1 (0) |
| Comoros | Bovine | 3/5 (60) |
|  | Ovine | 8/12 (66.6) |
|  | Goat | 9/18 (50) |
| Vaccinees | Ovine | 2*/8 (25) |

[a] *, antibodies were detected at day 79 but not at day 21 postvaccination.

velop an antibody response against NSs, a panel of sera was collected from different animal species (bovines, goats, and sheep) in different localities (Table 1). This allowed us to take into account the possible variable immune responses between animal species and to consider possible genetic variability of NSs in the circulating strains. These sera were obtained during surveys performed in Djibouti in 2000, in Mayotte in 2008, and in Grande Comore in 2010. All the sera used here had previously tested positive using a BDSL commercial kit, and their status was confirmed using homemade viral antigens. Most of them had a high antibody titer against RVFV antigen (mostly N antigen).

In the first attempt, the sera were assayed by indirect ELISA, using NSs antigen coated on the wells and serial 2-fold serum dilutions starting at 1:100. Under these conditions, less than half of the sera reacted (weakly) at low dilutions (not shown). In addition, when the test was performed with higher serum concentrations, unspecific reactions were obtained, making interpretation difficult. Given that serum concentrations as high as 1:50 to 1:12.5 should be tested, we decided to switch to cELISA. Figure 3A shows the results obtained with several sera representative of different types of responses. Sera were considered negative for NSs-specific antibodies when none of the serial dilutions were able to compete significantly or when only the highest concentration tested (1:12.5) led to (low) inhibition. This was the case for serum

3 from Mayotte and for sera 8 and 44 from Comoros. Sera were considered positive when at least three of the lowest serial dilutions (1:12.5, 1:25, and 1:50) led to significant inhibition, as illustrated for serum 5 from Mayotte and serum 18 from Comoros. For other positive samples, such as serum 7 from Djibouti or serum 21 from Comoros, the inhibition was higher than 50% at high concentrations, but the slope of the curve was relatively sharp, reaching the background value at a dilution of 1:100 or 1:200. Other samples, such as serum 1 from Mayotte, reacted positively on a wide range of dilutions, with a dose response similar to the one obtained with hyperimmune mouse serum (Fig. 2). As expected, none of the RVFV-negative samples competed in the NSs-based cELISA. Table 1 summarizes the complete data set, which shows that 55.5% of RVFV-infected animals ($n = 54$) elicited an immune response against NSs, corresponding to 56.5% of bovines ($n = 23$), 47.4% of goats ($n = 19$), and 66.6% of ovines ($n = 12$). However, it was difficult to evaluate if this slight difference between animal species was significant because of the relatively small number of animals of each species. Also, sera available from the different locations were not large enough for a statistical evaluation of the effects of the local RVFV strains.

**Analysis of RVFV-infected animal sera by Western blotting.** The absence of antibodies against NSs in approximately half of the sera tested could be explained by the lack of sensitivity of the cELISA and/or because animals did not develop NSs-specific antibodies. Therefore, we tested sera selected as positive or negative according to cELISA by using Western blotting. To avoid ambiguous results due to an eventual high background, each serum sample was tested against extracts from cells infected with the ZH548 strain of RVFV and two negative controls: uninfected Vero cells to detect unspecific antibodies and cells infected with ZH-DelNSs, a recombinant ZH548 virus in which the NSs sequence was entirely deleted (3). Remarkably, most sera gave a low background in Western blots, and the specific bands corresponding to N and NSs proteins were clearly distinguishable (Fig. 3B). Interestingly, sera found to be positive by cELISA (sera M#1, M#7, Com#18, Com#21, and Dj#7) reacted positively in Western blots against
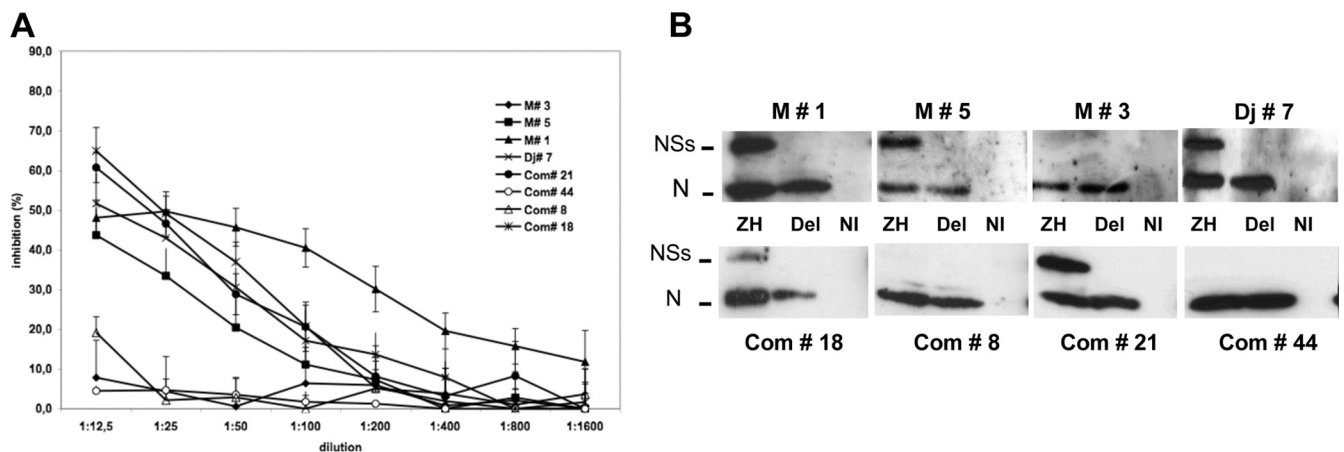


**FIG 3** Detection of NSs antibodies in naturally infected animals. (A) Serum samples from field animals were assayed by cELISA as described in the legend to Fig. 2C. Titration was performed using 2-fold serial dilutions. The remaining free epitopes were detected with NSs-specific rabbit antibodies, which were revealed with anti-rabbit HRP conjugate. (B) The serum samples titrated in panel A were tested in Western blots against extracts of noninfected Vero cells (NI) or cells infected with the RVFV ZH548 strain (ZH) or the RVFV ZH-DelNSs strain (Del; identical to ZH548 except for a complete NSs gene deletion). The positions of NSs and N are indicated.
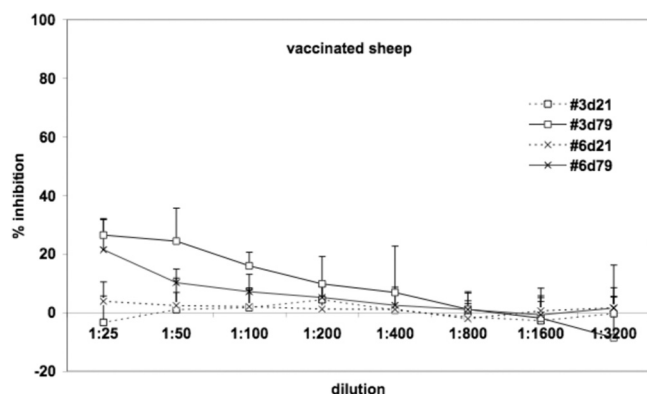
**FIG 4** Titration curve of NSs antibodies detected in vaccinated sheep. Eight sheep were vaccinated with the Smithburn strain of RVFV as recommended by the producer (Onderstepoort Biological Products). Antibodies against NSs were assayed by cELISA, and the 2 sera positive at day 79 after vaccination (sheep 3 and 6) were titrated, as well as the corresponding sera collected at day 21.

NSs as well as against N, used as an internal control. All negative samples by cELISA (sera M#3, Com#8, and Com#44) reacted only with the nucleoprotein, not with NSs (Fig. 3B). These results, as well as those obtained with other sera, established an excellent correlation between the results obtained by cELISA and Western blotting and confirmed the variable responses against NSs in animals naturally infected by RVFV.

**Production of antibodies against NSs results from a late immune response.** To understand the kinetics of the immune response against NSs, we decided to test animal sera positive for RVFV-specific IgM, as this is a sign of recent infection, since IgM persists for only 2 to 3 months after infection (28, 29). Since none of the preceding sera contained IgM, we obtained 10 RVFV IgM sera from bovines infected during the 2008-2009 RVFV outbreak in Madagascar (17). When these sera were assayed by cELISA or Western blotting, none of them was found to contain NSs-specific antibodies (not shown). To further investigate this result, we vaccinated sheep with the live attenuated Smithburn vaccine, which expresses a functional NSs protein, and collected sera at early (21 days) and late (79 days) time points after vaccination (M. Pépin et al., unpublished results). These animals were vaccinated subcutaneously with the dose recommended by the producer (Onderstepoort Biological Products). Interestingly, none of the sera collected at day 21 were found to be positive for NSs, although they were positive for other RVFV antigens, such as N. On day 79, only 2 of 8 (25%) vaccinated animals (sheep 3 and 6) developed NSs antibodies as revealed by cELISA. The corresponding sera exhibited a low but significant titration curve of NSs antibodies compared to the early sera from the same sheep collected at day 21 (Fig. 4) or compared to negative-control sera collected at day 0 (not shown). These data strongly suggest that the NSs-specific antibodies raised during vaccination with this attenuated strain of RVFV, if any, are elicited and detectable relatively late during infection. We do not know if this also applies during infection with virulent strains.

## DISCUSSION

The present data clearly indicate that the cELISA is sensitive and allows detection of low levels of NSs antibodies in RVFV-seropositive animals. In this context, it has a clear advantage over indirect ELISA, which requires dilutions of >1:100 to avoid unspecific reactivity that prevents detection of sera with low levels of antibodies. Nevertheless, approximately half of the sera of naturally infected animals were found to be negative for NSs antibodies as revealed by cELISA. This result is in agreement with other observations in our laboratory, as follows: (i) during experimental infection of mice, only a small fraction of the animals surviving infection with virulent RVFV showed detectable levels of antibodies against NSs; and (ii) the production of mouse polyclonal serum against recombinant NSs required several boosters to reach acceptable levels of antibodies, and only 58% of the mice (10 of 17 mice) elicited an NSs response. Altogether, the data strongly suggest that NSs is poorly immunogenic and induces antibodies in only a fraction of infected or vaccinated animals, from rodents to cattle, mostly in a late phase of the infection. This conclusion contrasts with that of a previously published paper (24). However, the previous study was based on an extremely small number of sera (4 goat and 4 human sera), thus precluding extrapolation to a large population, and the authors noted that sera generally exhibited low antibody titers. Although our analysis was also carried out on a limited sample, due to difficulties in obtaining RVFV-infected animal sera, it shows variation in the immune response against NSs, with correlation between cELISA and Western blot results. From a fundamental point of view, it would be interesting to investigate further the mechanism behind this inconstant antibody response against NSs and to evaluate if it could be of any importance in protection. Whatever the answer, the NSs positivity in only approximately 50% of infected animals raises questions regarding the use of NSs-specific antibodies as criteria to distinguish naturally infected animals from those vaccinated with NSs-defective RVFV vaccines. Even though such a DIVA test does not appear to be valid at the individual level, it may be envisaged at the herd level for a reasonable number of animals.

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